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FULL PAPER

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# Tetravalent *Pseudomonas aeruginosa* Adhesion Lectin LecA Inhibitor for Enhanced Biofilm Inhibition

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Dedicated to Professor *François Diederich*

A potent divalent ligand of the *Pseudomonas aeruginosa* adhesion lectin LecA was elaborated into a tetravalent version. A polyethylene glycol (PEG) spacer was introduced to link two divalent galactosides. Each of the two divalent ligands contained a rigid spacer with a central phenyl group that is bridged by the PEG moiety. The resulting tetravalent ligand was found to bind LecA in the nanomolar range involving all of its sugar (sub) ligands. Analytical ultracentrifugation studies clearly showed that the tetravalent ligand was capable of aggregation the LecA tetramers in contrast to the divalent ligands. The aggregator behavior was found to be of importance in *P. aeruginosa* biofilm formation inhibition. Despite the weaker affinity it was a considerably better biofilm inhibitor with half inhibitory values around the 28 micromolar range.

**Keywords:** *Pseudomonas aeruginosa*, multivalent carbohydrates, biofilm inhibition, ITC, analytical methods, analytical ultracentrifugation.

## Introduction

Lectins play important roles in the infections by *Pseudomonas aeruginosa*. This pathogen is one of the ESKAPE pathogens,[1] which is an indication of its resistance to antibiotics. Furthermore, the bacterium produces biofilms that make treatment even more difficult. It is clear that new treatments are needed.[2,3] Biofilms are problematic because they render antibiotics far less effective.[4] For this reason, it is very desirable to inhibit or even reverse the formation of biofilms using inhibitors.[5] Specifically for *P. aeruginosa*, biofilm inhibitors have been studied.[6] These were based on inhibition of the two bacterial extracellular lectins LecA and LecB.[7–10] Dendritic inhibitors of fucose-specific LecB were reported with an $IC_{50}$ of biofilm inhibition of 10 μM.[11] LecA binds to galactosides, and a tetravalent peptide dendrimer with four galactosides was shown to be an almost equally potent inhibitor of biofilm formation, while its lower valency counterpart was far less active.[12] Further increase beyond tetravalent did not lead to additional gains.[13] A covalent LecA inhibitor was shown useful for biofilm imaging.[14] Besides its role in biofilm formation, the LecA adhesion lectin is also deemed responsible for adhesion and invasions and causing lung injury. As such it is considered as an important target for intervention.[15] In this context, numerous groups have produced LecA inhibitors, and considering the fact that LecA is present as a tetramer, addressing this multivalency aspect has been pursued by numerous groups yielding nanomolar multivalent inhibitors of various architectures.[16–21] We have chosen to make divalent ligands, aiming to bridge two nearby sites of the LecA tetramer using a chelation mechanism.[22,23] The chelation-type divalent binding was confirmed by X-ray crystallography of **1a** bound

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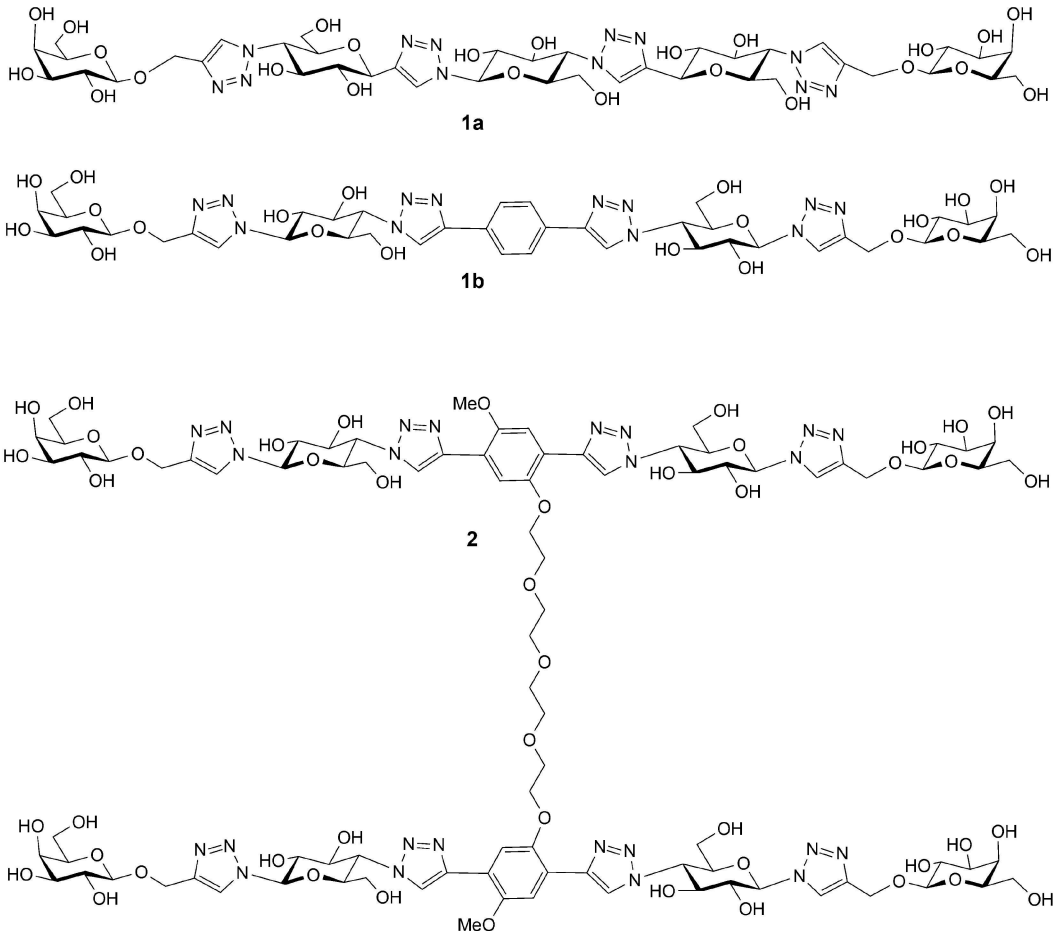



**Figure 1.** Structures of di- and tetravalent of *Pseudomonas aeruginosa* adhesion lectin LecA.

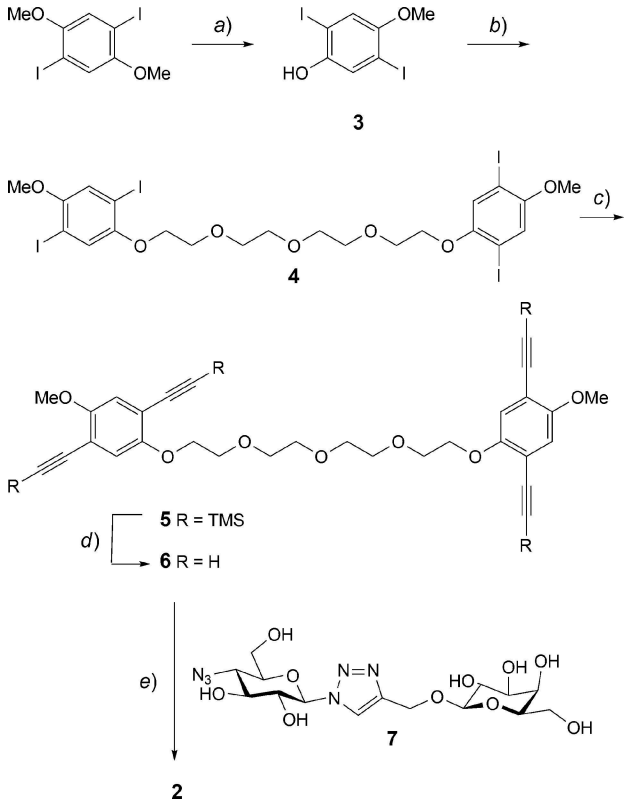
to LecA.[24] Recent further optimization of **1a** ($K_d$ 28 nM) revealed compound **1b** as a slightly more potent inhibitor with a $K_d$ of 12 nM (*Figure 1*).[25] Previously, it was found that divalent ligand **1a** was able to inhibit biofilm formation, but only at relatively high concentration (*ca.* 50% inhibition at 150 μM).[24] Based on the mentioned studies that a higher valency is beneficial for biofilm inhibition,[12] we decided to make a tetravalent version of **1b**, that would be able to crosslink LecA proteins, a feature that seems of importance in the inhibition of biofilm formation.

## Results and Discussion

For the design of the tetravalent ligand **2**, compound **1b** was used as the starting point. The central phenyl group was taken as the point of attachment of the linking moiety. While potent divalent binding of **1b** was achieved by using a rigid spacer, it was deemed beneficial to use a flexible PEG spacer for **2** to allow the cross-linking to proceed without constraints. A tetra-ethylene glycol unit was chosen for this purpose. The synthesis (*Scheme 1*) started with 1,4-diiodo-2,5-dimethoxybenzene which was selectively mono-deprotected by $BBr_3$ to give **3**.[26] Tetra-iodo compound **4** was obtained by linking two molecules of **3** with tetraethylene glycol ditosylate. A subsequent *Sonogashira* coupling yielded compound **5** with four TMS protected alkyne groups. Deprotection with potassium carbonate deprotection gave **6** with free alkynes. CuAAC Conjugation with azide **7**[25] provided final product **2** which was isolated in pure form after preparative HPLC.

The tetravalent ligand was first evaluated by ITC, in order to see whether the binding to LecA was still intact and not inhibited by the dimerization. The $K_d$ determined for **2** was 59 nM (*Table 1*). This indicates that the binding affinity in comparison to **1a** ($K_d$ 28 nM) and **1b** ($K_d$ 12 nM) is slightly reduced despite its statistical advantage of having twice the number of galactosides. Nevertheless, the affinity is still in the




**Scheme 1.** *a*) $BBr_3$, $CH_2Cl_2$, r.t., 78%. *b*) Tetraethylene glycol ditosylate, NaH, DMF, 120 °C, 70%. *c*) Ethynyltrimethylsilane, Pd $(PPh_3)_2Cl_2$, CuI, $Et_3N$, 87%. *d*) $K_2CO_3$, $MeOH/CH_2Cl_2$, 90%; *e*) $CuSO_4 \cdot 5\,H_2O$, Na-ascorbate, $DMF/H_2O$ 1:1, 100 °C, 1.5 h, 20%.

**Table 1.** Binding studies (ITC) and biofilm formation inhibition studies.

| Compound[a] | $K_d$ [nM][a] | $n$[b] | Biofilm inhibition[c] |
|---|---|---|---|
| **1a** | 28[23] | 0.55 | 50% at 150 μM[24] |
| **1b** | 12 | 0.41 | n.d.[d] |
| **2** | 59 | 0.21 | 46% at 28 μM |

[a]Isothermal-titration microcalorimetry (ITC). [b]Stoichiometry. [c] Inhibition and dispersal of *Pseudomonas aeruginosa* strain PA01 biofilms. [d]Not determined.

nanomolar range, clearly indicative of a strong multivalency effect. For comparison, the $K_d$ of the monovalent ligand **7** is 6.2 μM. For divalent ligands, the stoichiometry ($n$) as obtained from the ITC experiment, is typically around 0.5, indicative of a 2:1 binding with one inhibitor binding to two LecA subunits.[23] In the case of **2**, the $n$ value is close to 0.25, which indicates that each ligand binds to four LecA subunits. Considering that it is geometrically not possible for one molecule to bind to all four binding sites of a LecA tetramer, compound **2** must bind to at least two tetramers and will likely induce protein aggregation.[24,27]

Aggregation behavior of the multivalent ligand may be a factor in its biofilm blocking properties, therefore, we studied this behavior with analytical ultracentrifugation. Analysis of the sedimentation profiles yielded distributions of sedimentation coefficients for LecA with divalent ligand **1b** as shown in *Figure 2,a* and for LecA with tetravalent ligands **2** as

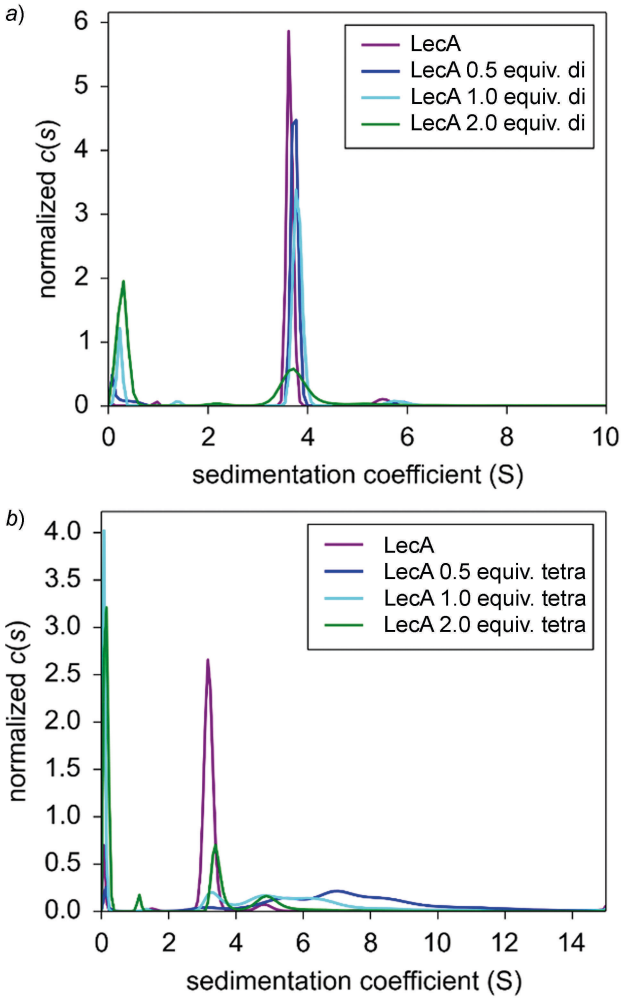



**Figure 2.** The *c*(s) distribution models for all measured samples *a*) for the LecA + **1b** and *b*) for LecA + **2** (with 5% DMSO added). *c*(s) is normalized on the total peak areas.

shown in *Figure 2,b*. A solution of pure LecA contained a species with a sedimentation coefficient of 3.8 S, corresponding to a mass of 50 kDa for the protein for LecA in the 'normal' buffer and a sedimentation coefficient of 3.5 S (54 kDa) for LecA in the presence of 5% DMSO ('DMSO-buffer'). The small differences might

be caused by some configurational change when DMSO is added to the buffer (differences in solvent density and solvent viscosity were accounted for). The friction factor of LecA in the 'DMSO-buffer' was higher than in the 'normal' buffer, so the shape of LecA in 'DMSO-buffer' deviated more from a sphere than LecA in 'normal buffer'. The obtained masses are in accordance with the predicted mass of 51 kDa for the normal LecA tetramer. A second peak only contributing *ca.* 4% of the signal had a value of 5.8 S (94 kDa) and 5.4 S (101 kDa) for LecA with and without DMSO respectively. These second peaks can be attributed to a small amount of the LecA tetramers dimerizing.

With the addition of divalent **1a**, no change in the signal was observed (*Figure 2,a*). The same peak at 3.7–3.8 S for the LecA protein was seen as well as another peak at about 5.7–6.1 S contributing 3–4% of the signal. The lack of any significant increase in the larger species indicates that the **1b** does not crosslink the LecA proteins in solution, a result consistent with previously measured **1a**.[24] With increasing amounts of **1b**, the peak at very low S is increasing in prominence and can clearly be attributed to the ligand. Addition of tetravalent **2** causes clear changes. Addition of 0.5 equivalent of ligand resulted in the formation of larger aggregates at 5.8 S, 9.2 S, and 17.3 S (molecular weights of 114, 229, and 586 kDa). These peaks might be due to dimers, tetramers, and even larger aggregates (*ca.* 10 proteins). When more ligand is added, the aggregates break up somewhat into smaller species, and for 2.0 equivalents of **2**, there are mainly monomers and dimers (apart from the pure ligand peaks at very low S) with sedimentation coefficient at 3.8 S and 5.7 S. At this amount of ligand the amount of dimer is increased from less than 5% (free LecA) to *ca.* 40%.

With the increased degree of aggregation, we studied the effect of **2** on biofilm formation inhibition and dispersion as described before (*Figure 3*).[6] For comparison, divalent ligand **1a** previously showed 50% biofilm formation inhibition at 164 μg/mL (150 μM, *Table 1*), using the same assay.[24] Tetravalent **2** was indeed more potent, with 46% biofilm formation inhibition at 64 μg/mL (28 μM). Similarly, in the biofilm dispersion assay, 50% dispersion is reached at the same concentration point. While this is a significant improvement over the divalent ligand, dendritic galactoside-linked peptides were previously shown to be more potent inhibitors with full inhibition at 20 μM.

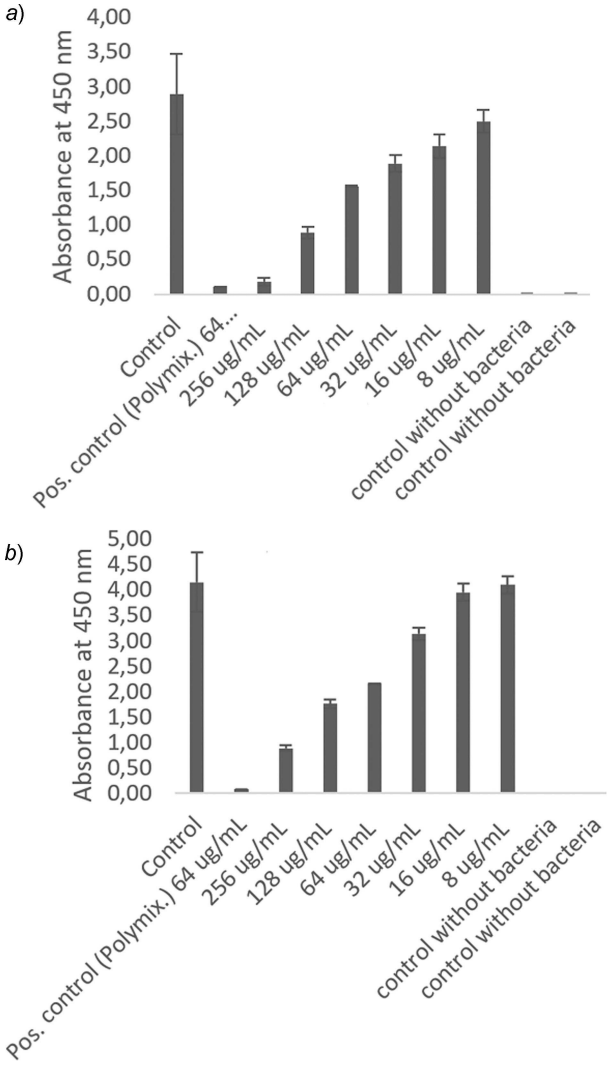



**Figure 3.** Inhibition (*a*) and dispersal (*b*) of *Pseudomonas aeruginosa strain* PA01 biofilms by various concentrations of **2**, *versus* positive control polymyxin (64 μg/ml). For inhibition, biofilms were grown on microtiter plates for 24 hours at 37 °C in the presence of the indicated compounds, and viable biofilms were stained with WST-8/PE. For dispersal, biofilms were first grown on microtiter plates for 24 hours at 37 °C in the absence of any compounds, planktonic bacteria were removed, and the biofilms were incubated with compounds for another 24 hours. Viable biofilms were stained with WST-8/PES.

## Conclusions

The synthesis of a dimerized divalent ligand **2** for LecA was successful by linking a PEG spacer to the central phenyl ring. The final CuAAC of four extended and fully deprotected ligands, followed by preparative HPLC purification proved to be the best method for the synthesis of **2**. ITC was applied as the most widely used method for affinity determination for LecA. The

$K_d$ of tetravalent **2** was higher in comparison with **1a** and **1b**. With a $K_d$ of 59 nM tetravalent **2** still ranks among the most potent LecA ligands. Its stoichiometry (*n*) being close to 0.25, indicating that all four galactoside ligands were engaged in binding, which for geometric reasons requires an aggregation mechanism. This was evaluated and confirmed by analytical ultracentrifugation. For **2**, clearly larger aggregates were formed in contrast to the monovalent **1a**[24] and **1b**. A concentration dependent *P. aeruginosa* biofilm formation inhibition and biofilm dispersion was both observed for tetravalent **2**. The inhibition occurred at a significantly lower concentration than previously determined for **1a**, however more potent tetravalent galactosides were reported whose peptidic backbone may contribute to their potency.[6] Considering that LecA binding occurred in the nanomolar range, while biofilm inhibition occurred in the micromolar range, it is clear that the later phenomenon is not only controlled by LecA binding. Nevertheless, compounds as presented here may be promising components of a multicomponent antibacterial solution with prophylactic properties, or, for example, based on synergy with antibiotics.[6]

## Experimental Section

*General*

Unless stated otherwise, chemicals were obtained from commercial sources and were used without further purification. Compound **3** was synthesized following literature procedures.[26] Solvents were purchased from *Biosolve* (Valkenswaard, The Netherlands). All moisture-sensitive reactions were performed under a nitrogen atmosphere. Anhydrous THF was dried over Na/benzophenone and freshly distilled prior to use. All the other solvents were dried over molecular sieves 4 Å or 3 Å. TLC was performed on *Merck* precoated *Silica 60* plates. Spots were visualized by UV light, 10% $H_2SO_4$ in MeOH and triphenylphosphine in THF followed by ninhydrin. Microwave reactions were carried out in a *Biotage* microwave initiator (Uppsala, Sweden). The microwave power was limited by temperature control once the desired temperature was reached. Sealed vessels of 2–5 mL were used. Analytical HPLC runs were performed on a *Shimadzu* automated HPLC system with a reversed-phase column (*Alltech*, *C8*, 90 Å, 5 μm, 250 × 4.6 mm, Deerfield, IL, USA) that was equipped with an evaporative light scattering detector (*PLELS 1000*, *Polymer Laboratories*, Amherst, MA, USA) and a UV/Vis detector operating at 220 nm and 254 nm. Preparative HPLC runs were performed on an *Applied Biosystems* workstation. Elution was effected by using a linear gradient of 5% MeCN/0.1% TFA in $H_2O$ to 5% $H_2O$/0.1% TFA in MeCN. $^1$H-NMR, HSQC, COSY (400 MHz, 500 MHz) and $^{13}$C-(100 MHz, 126 MHz) were performed on a *Varian G-300* spectrometer. Electrospray Mass experiments were performed in a *Shimadzu LCMS QP-8000*. High resolution mass spectrometry (HR-MS) analysis was performed using an *ESI-QTOF II* spectrometer (*Bruker*, Billerica, USA) and *Applied Biosystems 4700* MALDI TOF/TOF instrument.

**1,1′-{Oxybis[(ethane-2,1-diyl)oxyethane-2,1-diyloxy]}bis(2,5-diiodo-4-methoxybenzene)** (**4**). 2,5-Diiodo-4-methoxyphenol (250 mg, 665 μmol) was dissolved in DMF (3 mL), then NaH (16.8 mg, 698 μmol) was added. The mixture was stirred for 30 min. Then, tetraethylene glycol ditosylate (167 mg, 333 μmol) was added, the mixture was stirred at 120 °C overnight. $CH_2Cl_2$ (10 mL) was added, and the mixture was washed by water and brine, dried with sodium sulfate, and purified by column chromatography (petroleum ether/ethyl acetate 1:1) to afford the compound as a white solid (424 mg, 466 μmol, 70%). $^1$H-NMR (500 MHz, $CDCl_3$): 7.23 (*s*, 2 H); 7.15 (*s*, 2 H); 4.08 (*t*, $J$ = 4.9, 4 H); 3.89–3.84 (*m*, 4 H); 3.80 (*s*, 6 H); 3.76 (*dd*, $J$ = 5.8, 3.6, 4 H); 3.69 (*dd*, $J$ = 5.7, 3.6, 4 H). $^{13}$C-NMR (126 MHz, $CDCl_3$): 153.7; 153.0; 123.9; 121.4; 86.7; 85.5; 77.4; 77.2; 76.9; 71.3; 71.0; 70.5; 69.8; 57.3. HR-ESI-MS (Q-TOF): 927.8209 ($C_{22}H_{30}I_4O_7N^+$, $[M+NH_4]^+$; calc. 927.8201).

**{Oxybis[(ethane-2,1-diyl)oxyethane-2,1-diyloxy-(5-methoxybenzene-2,1,4-triyl)di(ethyne-2,1-diyl)]}-tetrakis(trimethylsilane)** (**5**). Compound **4** (211 mg, 165 μmol), $Pd(PPh_3)_2Cl_2$ (25 mg, 35 μmol), and CuI (6.6 mg, 35 μmol, 0.15 equiv.) were filled to the flask and degassed for 1 h. Then the degassed $Et_3N$ (6 mL) was added, and finally, ethynyltrimethylsilane (114 mg, 1.2 mmol) was added through a syringe. The resulting mixture reacted at room temperature overnight. $Et_3N$ was removed under vacuum. $CH_2Cl_2$ was added to extract the product, and it was further washed by water and brine and dried by sodium sulfate. After column purification (petroleum ether/ethyl acetate 8:1), the pure compound was obtained as a brown solid (114 mg, 144 μmol, 87%). $^1$H-NMR (500 MHz, $CDCl_3$): 6.93 (*s*, 2 H); 6.88 (*s*, 2 H); 4.12 (*t*, $J$ = 4.9, 5 H); 3.86 (*t*, $J$ = 4.9, 4 H); 3.82 (*s*, 6 H); 3.79–3.76 (*m*, 4 H); 3.70–3.65 (*m*, 4 H); 0.25 (*d*, $J$ = 7.7, 36 H). $^{13}$C-NMR (126 MHz, $CDCl_3$): 154.6; 153.8; 118.4; 115.8; 114.3;

113.5; 101.1; 100.8; 100.6; 100.5; 77.4; 77.2; 76.9; 71.3; 70.9; 69.9; 69.7; 56.5; 0.2; 0.1. HR-ESI-MS (Q-TOF): 808.3929 ($C_{42}H_{66}NO_7Si_4^+$, $[M+NH_4]^+$; calc. 808.3916).

**1,1′-{Oxybis[(ethane-2,1-diyl)oxyethane-2,1-diyloxy]}bis(2,5-diethynyl-4-methoxybenzene)** (**6**). To a solution of compound **5** (156 mg, 197 μmol) in MeOH/$CH_2Cl_2$ (3:1, 4 mL), $K_2CO_3$ (110 mg, 790 μmol) was added. The mixture reacted at room temperature for 1 h. After removal of the solvents, $CH_2Cl_2$ was added to extract the product. Then, it was washed with water and brine and dried with sodium sulfate. After removal of the $CH_2Cl_2$, the residue was purified by column chromatography to afford the product as a brown solid (90 mg, 177 μmol, 90%). $^1$H-NMR (400 MHz, $CDCl_3$): 7.02 (*s*, 2 H); 6.95 (*s*, 2 H); 4.15 (*dd*, *J* = 5.5, 4.3, 4 H); 3.89–3.85 (*m*, 11 H); 3.78–3.74 (*m*, 4 H); 3.70–3.67 (*m*, 4 H); 3.38 (*s*, 1 H); 3.35 (*s*, 1 H). $^{13}$C-NMR (101 MHz, $CDCl_3$): 154.8; 153.9; 118.7; 116.0; 113.8; 112.7; 83.1; 82.9; 79.8; 79.7; 77.5; 77.2; 76.8; 71.2; 70.9; 69.8; 69.7; 56.5. HR-ESI-MS (Q-TOF): 520.2338 ($C_{30}NH_{34}O_7^+$, $[M+NH_4]^+$; calc. 520.2335).

*Tetravalent Ligand* **2**. To a solution of compound **6** (4.9 mg, 9.7 μM), compound **7**[25] (21.6 mg, 48.3 μM) in DMF (0.9 mL), an aqueous solution (25 μL) of $CuSO_4 \cdot 5H_2O$ (2.4 mg, 9.7 μM) and Na-ascorbate (1.9 mg, 9.7 μM) was added. Finally, TBTA (5 mg, 9.7 μM) was added. The mixture was reacted under microwave irradiation at 100 °C for 2 h. Then, Copper-absorbent (*Cuprisorb*) was added, filtered, and the solvent was removed under reduced pressure. The residue was purified by preparative HPLC to afford the product as white solid (5 mg, 2 μM, 20%). $^1$H-NMR (500 MHz, ($D_6$)DMSO + 1 drop $D_2O$): 8.56 (*s*, 2 H); 8.48 (*s*, 2 H); 8.40 (*s*, 2 H); 8.38 (*s*, 2 H); 7.92 (*s*, 2 H); 7.90 (*s*, 2 H); 5.91 (*t*, *J* = 8.10, 4 H); 4.89 (*d*, *J* = 11.96, 4 H); 4.68–4.63 (*m*, 8 H); 4.38–4.34 (*m*, 4 H); 4.28–4.22 (*m*, 12 H); 4.02–3.99 (*m*, 10 H); 3.91–3.89 (*m*, 4 H); 3.69–3.56 (*m*, 20 H, overlapped with deuterated solvents); 3.42–3.40 (*m*, 4 H); 3.34–3.29 (*m*, 10 H); 3.26–3.24 (*m*, 2 H;) 3.13–3.11 (*m*, 4 H). $^{13}$C-NMR (126 MHz, ($D_6$)DMSO + 1 drop $D_2O$, extracted from HSQC): 125.5; 124.8; 124.1; 111.3; 109.8; 103.3; 87.6; 77.6; 75.7; 74.5; 73.7; 72.9; 70.9; 70.3; 70.2; 69.5; 68.6; 68.6; 62.2; 61.8; 61.8; 61.0; 60.2; 60.1; 60.1; 56.3. HR-ESI-MS (Q-TOF): 2295.8213 ($C_{90}H_{127}N_{24}O_{47}^+$, $[M+H]^+$; calc. 2295.8285).

*Isothermal Titration Microcalorimetry* (ITC)

The lectin LecA, obtained from *Sigma–Aldrich*, was dissolved in buffer (0.1 M *Tris*-HCl, 6 mM $CaCl_2$, pH 7.5) and degassed. Protein concentration (between 10 and 40 μM depending on the ligand affinity) was checked by measurement of optical density by using a theoretical molar extinction coefficient of 28,000. Carbohydrate ligands were dissolved directly into the same buffer, degassed, and placed in the injection syringe. ITC was performed using a *MicroCal Auto ITC200* (*Malvern*, Worcestershire, UK). LecA was placed into the 200 μL sample cell at 25 °C. A titration was performed with injections of carbohydrate ligands (2.5 μL) every 120 s. Data were fitted using the 'one-site model' using MicroCal Origin 7 software according to standard procedures. Fitted data yielded the stoichiometry (*n*), the association constant ($K_a$), the enthalpy ($\Delta H$) and the entropy of binding. The $K_d$ value was calculated as $1/K_a$, and *T* is 298 K.

*AUC Study of Aggregation Behavior of LecA with* **1b** *and* **2**

For the samples of LecA and divalent ligand **1b** a buffer of 0.1 M Tris and 6 mM $CaCl_2 \cdot 2\,H_2O$; pH = 7.5 was used (later called 'normal buffer'). With the software Sednterp the solvent density is calculated to be 1.0017 g/mL and the solvent viscosity 1.034 mPa s. Three mixtures were made just before start of the AUC measurement of 200 μL 40 mM LecA with 200 μL of 10 mM ligand, 20 mM ligand, or 40 mM ligand, and these were put in a vortex mixer for a few seconds.

For the samples of LecA and **2**, a buffer (0.1 M Tris and 6 mM $CaCl_2 \cdot 2\,H_2O$), containing 5% DMSO is used (called DMSO-buffer). Sednterp results combined with properties of DMSO, the solvent density was determined to be 1.0066 g/mL and the solvent viscosity 1.082 mPa s. Three mixtures were made just before the start of the AUC measurement of 200 μL 40 mM LecA with 200 μL of 5 mM ligand, 10 mM ligand, or 20 mM ligand and these were put in a vortex mixer for a few seconds. The 12 mm path length 2-sector aluminum centerpiece cells with sapphire windows, filled with about 380 μL sample and 400 μL buffer as a reference, were put in an *An60Ti* analytical rotor for a run in an *Optima XL–I* or *Optima XL-A* analytical ultracentrifuge (*Beckman Instruments*) at 50 krpm and at a temperature of 20 °C. Changes in solute concentration were detected by 500 absorbance scans measured at 280 nm over a period of 5–6 h.

Analysis and fitting of the data was performed using the software SedFit.[28] A continuous *c*(s) distribution model was fitted to the data, taking every 8th

scan. The resolution was set at 200 over a sedimentation coefficient range of 0.0–15.0 S. The frictional coefficient, the baseline and the raw data noise were floated in the fitting. The meniscus of the cell path was also floated after initial estimations from the raw data (the bottom remained fixed).

*Biofilm Inhibition and Dispersal on Polystyrene Microtiter Plates*

96-well sterile, U-bottomed polystyrene microtiter plates (*TPP* Switzerland) were prepared by adding 200 μL of sterile deionized water to the peripheral wells to decrease evaporation from test wells. Aliquots of 180 μL of culture medium (*M63* medium broth enriched with 20% glycerol, 1 M $MgSO_4$, and potassium hydroxide) containing desired concentration of the test compound were added to the internal wells. Compound containing solutions were sterile filtered (pore size 0.22 μM) prior to addition to the wells. Inoculum of *Pseudomonas aeruginosa* strain PAO1, was prepared from 5 mL overnight culture grown in LB broth overnight at 37 °C and 180 rpm shaking. Aliquots of 20 μL of overnight cultures, pre-washed in 0.25% (*w/v*) nutrient broth and normalized to an *OD600* of 1.5, were inoculated into the test wells. Plates were incubated in a humid environment for 24–25 h at 37 °C under static conditions. Wells were washed twice with 200 μL sterile deionized water before staining with 200 μL M63 broth containing 0.5 mM WST-8 and 20 μM phenazine ethosulfate (PES) for 4 h at 37 °C under static conditions. Afterwards, the well supernatants were transferred to a polystyrene flat bottomed 96-well plate (*TPP* Switzerland) and the absorbance was measured at 450 nm with a plate reader (*SpectraMax250* from *Molecular Devices*).

For biofilm dispersal, a biofilm was formed as described above, but in the absence of compound for 24 h. Wells were washed twice with 200 μL sterile deionized water before adding 200 μL 0.25% (*w/v*) nutrient broth containing the desired concentration of compound. Compound-containing solutions were sterile filtered (pore size 0.22 μm) prior to addition to the wells. After another 24 h of incubation at 37 °C under static conditions, the well supernatants were removed and the wells were washed twice with 200 μL sterile deionized water. The biofilm was stained with 200 μL of 0.25% (*w/v*) nutrient broth containing 0.5 mM *WST-8* and 20 μM phenazine ethosulfate (PES) for 4 h at 37 °C under static conditions. The resulting absorbance was measured as in the biofilm inhibition experiment.

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## Author Contribution Statement

*G. Y.* and *R. J. P.* designed the experiments, analyzed the data, and wrote the article. *G. Y.* performed the synthesis and binding, *D. M. E. T.-W* performed and analyzed the analytical ultracentrifugation work.

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